Single-Use Fluoropolymer Bags for Efficient High-Density Cell Banking

Entegris

INTRODUCTION

The need for rapid development and scale-up of vaccines and therapeutics has never been greater. But conventional seed train processes used to amplify cells from cell cultures are inefficient and subject to risk. They start with relatively small quantities of cells, requiring weeks of processing and multiple transfer steps. Cell cultures are typically stored in vials ranging from 1 to 5 milliliters (mL) in size. The cells must remain healthy throughout the process of cold storage, thawing, amplification, and inoculating a bioreactor.

Conventional cell culture storage uses cylindrical cell cryopreservation containers accommodating between 12 and 18 vials. The containers are filled with isopropyl alcohol to control the freezing rate. Freezing cells in individual vials achieves the goals of maintaining cell viability after long-term cold storage, but it comes with some drawbacks. The typical seed train involves an open system of multiple manual steps to transfer cells from vials to successively larger flasks to bioreactors (Figure 1). Every time cells are transferred to a larger flask, there is a risk of contamination. Each batch requires a new seed culture, introducing risks of inconsistent cell quality between batches.

This multistep process is expensive and time-intensive, taking weeks to amplify the cell count to a high enough level to inoculate a production bioreactor. There is, therefore, a need for a more efficient, streamlined approach that will speed up the cell amplification process and reduce cost while maintaining high cell quality. Improving efficiency requires increasing the volume of media in cell cultures, the cell density, or both, and reducing the number of process steps.

Research on high-density cell banking has demonstrated its ability to increase the density in cell cultures from 10 million cells per mL up to 100 million cells per mL.[1] This approach reduces the number of steps in the seed train, shrinking processing time from weeks down to days.[2] By eliminating manual transfer steps, high-density cell banking minimizes contamination risk. Instead of storing frozen cell cultures in individual vials, high-density cell banking relies on larger vessels.

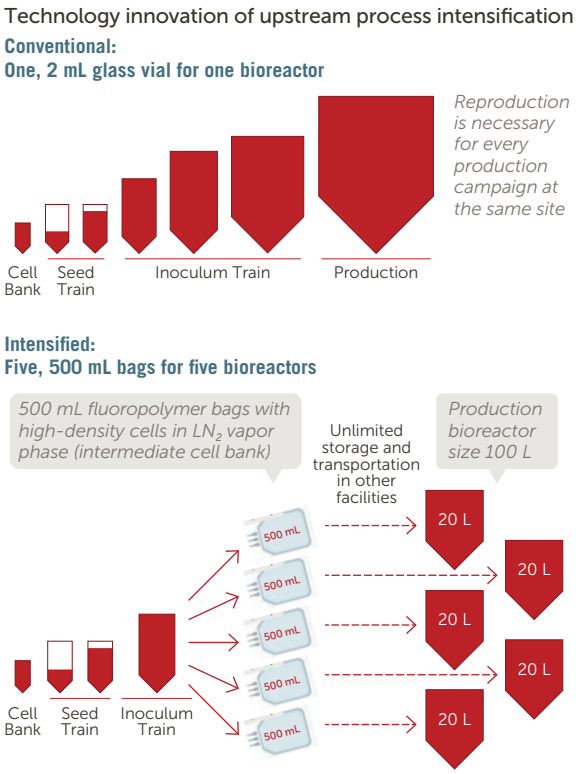


Figure 1. Conventional seed train process versus intensified approach, reduces processing time by several weeks.

When replacing cell cryopreservation containers with another storage method, the cells must be protected throughout the freezing, storage, thawing, and transfer processes. In addition to saving time by compressing the seed train, the chosen method should meet the following requirements:

- Control the freezing rate to near 1°C per minute down to at least -40°C
- Accommodate a variety of standard permeating and non-permeating cryoprotectants
- Enable high-cell density
- Survive freezing to -150°C for long-term cold storage
- Achieve a thawing rate that equals or exceeds industry standards
- Achieve cell viability of at least 90% at the end of the process
- Scale sufficiently for seeding of large (20 to 100 liter) bioreactors
- Be cost-effective and reproducible

Single-use 2D storage bag assemblies made from fluoropolymers offer a solution that increases both cell culture volume and cell density per mL while preserving cell viability. This white paper explains how these bags perform during freezing, storage, and thawing steps and why they present an efficient alternative to vials in a variety of upstream cell banking applications.

RETHINKING CELL FREEZING

Freezing and thawing is stressful for all cells, but by implementing best practices it is possible to optimize cell viability. Freezing rate is the key variable that determines the cell physiology and survival rate after thawing. It is important to maintain a cooling rate close to -1°C per minute during the initial freezing process down to at least -40°C.[3] If cooling is too slow, excessive generation of ice crystals around the cells induces mechanical stress and cell morphology suffers. Cooling too rapidly can cause the formation of ice crystals inside the cells. When cooling further for media vitrification and long-term cryogenic storage, the rate of temperature decease is not critical.

Conventional cell cryopreservation containers control the freezing rate of the cells passively by insulating the vials. Filling the container with isopropyl alcohol (IPA) or other alcohol-based solvents allows the cells inside the vials to be cooled at an acceptable rate simply by placing the container into a fixed-temperature freezer.

Single-use storage bags present an alternative method for freezing cell cultures. While many such bags have been on the market for decades, implementing them for cell banking applications has proved challenging.

Freezing rate, which is critical for success, has not often been well-controlled. Maintaining the integrity of the bag throughout the process is vital, and some commercial bags have experienced failures leading to microbial contamination. Not all products are compatible with the ultra-low storage temperatures that many applications require. The choice of bag and tubing material plays an important role in determining suitability for upstream bioprocessing.

Entegris has been working with university laboratories at CPE Lyon and IUT Lyon to evaluate single-use fluoropolymer bag assemblies for high-density cell banking. These bags are made from a single fluoropolymer layer that remains flexible at extremely low temperatures and is often tested down to -196°C.[4] The bags are available in a variety of sizes and can hold higher volumes of cells than an 18-vial cell cryopreservation container.

Experiments compared the performance of 50 mL and 500 mL bags to conventional cell cryopreservation container storage. Inserting the bag assembly into a metal cryoshell both protects the bag assembly and ensures maximum contact area with the metal for consistent cooling of the bag's contents. The cryoshells can be air-cooled from laboratory ambient down to below -40°C in a freezer, as is standard practice when using cell cryopreservation containers.

For relatively small bags, however, the cryoshell tends to cool the cell culture too rapidly when placed directly on a freezer shelf. To alleviate this issue, the cryoshell can be enclosed in an insulated cryobox, which serves a similar heat transfer function as the cell cryopreservation container. The cryobox, Figure 2, is lined with polyethylene (PE) foam rather than filled with IPA.

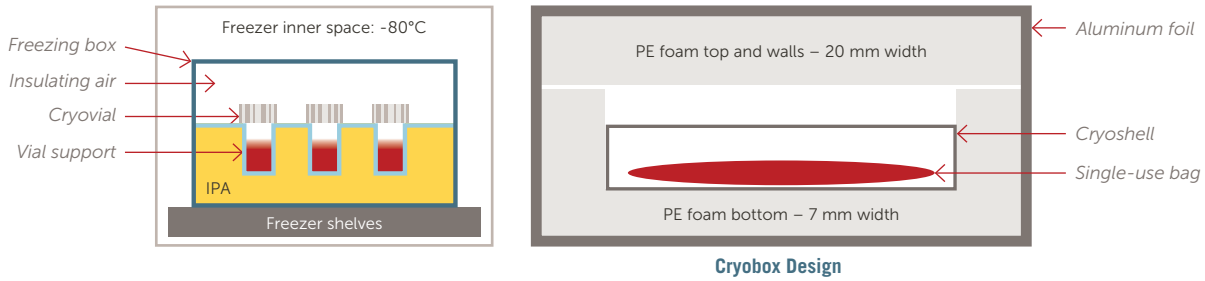


Figure 2. Cryobox/cryoshell combination for freezing 50 mL bags.

Testing has demonstrated the need for a cryobox when freezing cells in 50 mL bags. With the cryobox, the freezing rate remains within an acceptable range and similar to that achieved with vials, Figure 3. As the data show, fill volume aects the cooling rate. Volumes between 15 and 35 mL deliver the best results.

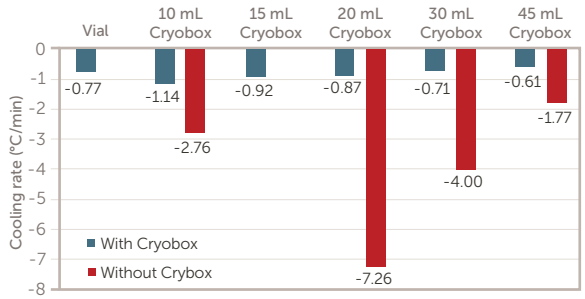


Figure 3. Comparison of the freezing rate for cells in vials and 50 mL bags with and without the cryobox. The freezing rate in the bag varies depending on the fill volume.

When moving to 500 mL bags, a lower fraction of the bag's contents is in contact with the metal surface of the cryoshell, decreasing the freezing rate. A near-optimum freezing rate can be achieved by placing the cryoshell directly on the freezer shelf and omitting the cryobox. The cooling profile does vary for different locations within the larger bag, as demonstrated by testing with thermocouples placed at multiple positions. But cooling profiles also vary between cell cryopreservation containers placed at different locations in a freezer. As with the smaller bags, the cooling profile for 500 mL bags depends on the fill volume.

General best practice for single-use bags fills them to between 50 and 70% of the bag's capacity. Doing so avoids problems associated with overfilling such as increased risk of leakage or rupture but does not address the stringent cooling rate requirements for freezing cells. The optimum fill rate for cell banking applications is much lower and must be determined based on the sensitivity of the cell line, cryoprotectant composition, and cell density. The chosen fill volume is important to consider when selecting the optimum bag size. While filling to 70% capacity is appropriate for bag integrity, it is likely too high a level for uniform cell freezing at or near -1°C per minute.

For short-term cold storage, up to one year, fluoropolymer bags filled with cell cultures can remain in a freezer set to -80°C. Long-term storage requires a lower temperature, which can be achieved by hanging cryoshells in the vapor phase of a liquid nitrogen tank. The vapor phase temperature of -150°C is below the typical media vitrification temperature of -120°C, allowing for a hard freeze.

CRYOPROTECTANT COMPATIBILITY

Cryoprotectants are required to eliminate ice crystal formation within the cell culture and thereby improve the cell survival during freezing. The ideal chemical reagent and concentration must be selected based on the type of cells and desired cell density.

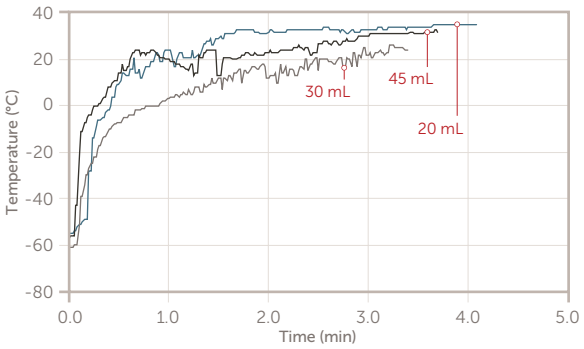
Cryoprotectants may be either permeating, meaning that the reactant permeates the cell membrane, or non-permeating. Permeating reagents such as dimethyl sulfoxide (DMSO) are more effective than non-permeating reagents, but they are toxic to cells at room temperature if the cryoprotectant concentration is too high. A washing step is often included to reduce cryoprotectant concentration after thawing and before introducing cells into the bioreactor. Washing is not required when using non-permeating reagents because these sugars or starches are nontoxic, but such cryoprotectants may not sufficiently protect against cell damage from freezing.

Fluoropolymer storage bags have been evaluated using both DMSO and a DMSO-free commercial cryoprotectant. Results show that the bags are compatible with both types of cryoprotectant, giving laboratories the flexibility to choose whichever cryoprotectant chemical and concentration will work best for their cells. Measurements of cell viability, as defined by the cell population doubling rate after thawing, has demonstrated that freezing in bags is equal or superior to freezing in vials, with or without a washing step.

Increasing the initial cell density before freezing conveys two benefits. First, more cells are available for a given bag size and fill level. Second, if the amount of DMSO remains constant, the effective DMSO concentration is lower. This means that it should be possible to eliminate the cell washing step after thawing while maintaining sufficiently high cell viability. When transferring the cell media from 500 mL bags directly to a large bioreactor, the cryoprotectant becomes further diluted and negates potential toxicity. Viability data demonstrate that dilution works to protect cells from cryoprotectant-related damage. Eliminating the washing step further streamlines the process and reduces contamination risks, saving both time and expense.

RAPID THAWING

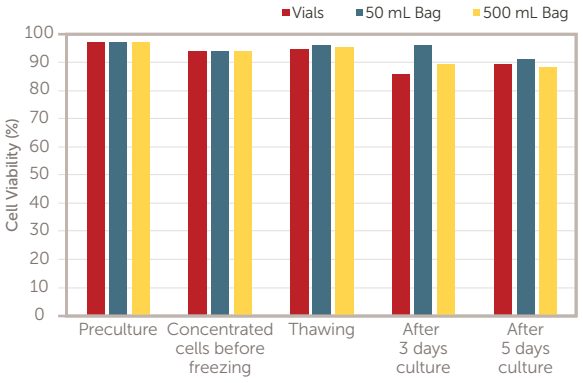
The thawing step must also be done properly to ensure cell viability. During thawing, it is best to ramp the frozen media up to ambient temperature as quickly as possible to minimize damage to cells. Incubation in a water bath set to 37°C is much faster than other methods, such as thawing in air or in a refrigerator. It is, therefore, the most common approach. Fluoropolymer single-use bags work well with water bath thawing. Manual agitation in the bath, which further increases the thawing rate, is especially effective for bags. The 50-mL bags thaw from -60°C in less than two minutes (Figure 4), which is much faster than thawing in vials.



Temperature record during thawing of bags in 37°C water bath at different volumes. Bags were taken out of Cryobox and Cryoshell before being immersed in water bath and manually agitated until complete disappearance of ice.

Figure 4. Thawing data for 50 mL bags.

Measurements of cell growth after thawing show better viability for cells frozen in bags than for vials (Figure 5). Freezing in bags improves homogeneity of the cell culture during freezing and allows for a faster thaw time, both of which are advantageous for cell survival.



Cell viability over experiment steps. Cells were harvested from a bioreactor (preculture) concentrated and resuspended as a single pool before distribution in vials (in Mr. Frosty), 50 mL bag (in Cryobox), and 500 mL (in Cryoshell only). Viability in culture was measured in shake flask cultures.

Figure 5. Cell viability before and after freezing for cells frozen in vials, 50 mL bags, and 500 mL bags.

SCALING TO HIGHER VOLUMES

High-density cell banking using fluoropolymer single-use bags for storage is easily scalable. Using a fed-batch amplification process, 500 mL bag with a cell density of 20 or 30 million cells per mL can directly seed a 20-L bioreactor with cell viability higher than that achieved using conventional methods of vials and shake flasks. Further scaling for large production bioreactors can be achieved by transferring multiple 500 mL bags to the bioreactor, further increasing the cell density, and filling each bag to the maximum level conducive to ideal cell freezing rate.

Experiments have verified that single-use fluoropolymer bags are an efficient freezing method that is ideal for short-term or long-term storage of classical biologics such as recombinant proteins and monoclonal antibodies (mAb). The process successfully amplifies cells, saving time and money while reducing the contamination risk inherent in conventional seed trains. Future extensions of this approach include cell and gene therapy applications.

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